# Annexin V perturbs or stabilises phospholipid membranes in a calcium-dependent manner

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**Abstract The potency of annexin V to transport $Ca^{2+}$ ions across phospholipid membranes was investigated, using large unilamellar phospholipid vesicles loaded with the $Ca^{2+}$ indicator fura-2. It was demonstrated that annexin V leaves the vesicle membranes intact when added in the presence of 1 mM $Ca^{2+}$. However, if the vesicles were first incubated with annexin V in the absence of $Ca^{2+}$, subsequent addition of $Ca^{2+}$ produced a fluorescence signal due to binding of $Ca^{2+}$ to fura-2. Centrifugation of the vesicle suspension immediately thereafter showed that this signal originated from the supernatant and not from the sedimented vesicles. Our results show that annexin V causes loss of vesicle integrity in the absence of $Ca^{2+}$, and leakage of trapped fura-2, rather than inward $Ca^{2+}$ transport. Bovine serum albumin or $Ca^{2+}$ concentrations higher than 2.5 mM also caused such fura-2 leakage. Apparently, calcium-dependent binding of annexin V to the membrane prevents aspecific membrane damage caused by this protein.**

*Key words:* Annexin V; Fura-2; Large unilamellar vesicle; Membrane damage; Calcium channel

## 1. Introduction

Annexin V was discovered in 1985 (then named Vascular Anticoagulant Protein) by our group as a protein in vascular endothelium with a strong anticoagulant effect [1]. In the presence of calcium it has a high binding affinity for membranes containing amino-phospholipids [2]. More recently it was claimed that its anticoagulent effect is based on the formation of large two-dimensional clusters of phospholipid-bound annexin V molecules that inhibit lateral diffusion of coagulation factors on the membrane surface [3], thereby reducing their collisional probability. From these studies it became apparent that annexin V binds peripherally to the membrane in the presence of calcium, and readily desorbs from the mmbrane for low calcium concentrations.

One of the roles proposed for annexin V is the formation of calcium channels in phospholipid bilayers. Using patch-clamp and dipping techniques, phospholipid bilayers were formed on micropipette tips and, after addition of annexin V to one side of these membranes, single-channel activity was measured in the presence of calcium [4,5]. Also, large unilamellar phospholipid vesicles (LUVs) loaded with fura showed fluorescence of the fura-calcium complex after addition of annexin V [6]. This was interpreted as formation of calcium channels promoting influx of calcium into the vesicles. Ultrastructural analysis has revealed a hydrophilic pore in the annexin V molecule that could serve as an ion-selective filter [5,7,8].

In view of the earlier mentioned peripheral binding and rapid desorption of annexin V, the formation of transbilayer channels of this protein seems surprising. Also, using black lipid membranes [9,10] of much larger surface area than membranes on micropipettes, we were unable to confirm calcium channel formation by annexin V in the presence of 1 mM calcium (H. Miedema, IMBS, Amsterdam, unpublished results).

Here we report the investigation of membrane perturbing properties of annexin V in the LUV system as described by Berendes [6]. The obtained results show that at low calcium concentrations annexin V perturbs membranes such that they become leaky. In contrast, at higher calcium concentrations annexin V promotes membrane integrity.

## 2. Materials and Methods

Human placental and recombinant annexin V were prepared as described respectively in references [1] and [11]. Bovine brain phosphatidyl-serine (PS) and bovine heart phosphatidyl-ethanolamine (PE) were obtained from Avanti Polar Lipids, Birmingham, AL, USA. Fura-2 (pentapotassium salt) was obtained from Molecular Probes Inc., Eugene, OR, USA. EGTA (ethylene glycol bis(2-aminoethyl ether)), HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulfonic acid), Triton X-100 (octyl pheroxy polyethoxy-ethanol), and bovine serum albumin (fatty acid free) were obtained from Sigma Chemical Co., St. Louis, MO, USA. All other reagents were from Merck, Darmstadt, Germany.

Large unilamellar vesicles (LUVs) were prepared as described in [12] and loading with fura-2 was performed as described in [6]. Briefly, a glass tube containing about 0.5 ml of chloroform with 2.7 $\mu$mol PS and 0.3 $\mu$mol PE was kept at 80°C and the solvent was evaporated by a slow stream of nitrogen gas, taking care not to disturb or agitate the solution. The remaining lipid film was rehydrated at 37°C with nitrogen saturated with water, and gently overlayed with 100 $\mu$l of HEPES buffer (5 mM; pH 7.4) with 180 $\mu$M EGTA and 160 mM sucrose, containing 100 $\mu$M fura-2. The suspension was kept at 37°C for 2 h without shaking or stirring. Hereafter, 100 $\mu$l of the same buffer without fura-2 was added and the suspension was centrifuged at 24°C for 10 min at 12,000 × *g* in a Beckman TL-100 ultracentrifuge. The supernatant was discarded and the pellet resuspended in 200 $\mu$l of HEPES buffer (10 mM; pH 7.4) with 200 $\mu$M EGTA and 180 mM sucrose. This slightly hyperosmotic buffer was used for resuspension in order to prevent swelling of the vesicles. Centrifugation and resuspension was repeated twice again in order to remove excess fura-2 and multilamellar phospholipid structures. The suspension thus obtained was used as a stock from which 40 $\mu$l at a time was taken to perform 5 experiments with the same vesicle preparation.

Fluorescence experiments were performed at 37°C, in a medium (2 ml) containing HEPES buffer (10 mM; pH 7.4) with 180 $\mu$M EGTA and 160 mM sucrose, with excitation and emission wavelengths of 340 nm and 500 nm, respectively, in an Aminco SPF-500 spectrofluorime-

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ter. In order to verify if increased fluorescence originated from the LUVs or from the buffer solution, centrifugations were again performed for 10 min at 12,000 × g.

## 3. Results

The effect of calcium (1 mM) and fura-2 concentration on fluorescence at 500 nm, after excitation at 340 nm, was measured in buffer with 180 μM EGTA and 160 mM sucrose. The results are presented in Fig. 1. and it was verified that addition of Triton X-100 (0.1%) and phospholipid (0.3 mM) had no influence on these data. Comparison of the increase in fluorescence intensity after addition of calcium in Fig. 1 with the total increase in fluorescence intensities measured in actual experiments after addition of Triton X-100 (see below) showed that about 3% of the 10 nmol of fura-2 present in the original 100 μl overlay was entrapped into the vesicles.

Exteral calcium concentrations higher than 2.5 mM caused loss of vesicle integrity. This is shown in Fig. 2, demonstrating that 1 mM of calcium had no effect, but 2.5 mM of calcium caused a large increase in fluorescence. Control experiments, in which the suspension was centrifuged, instead of adding Triton X-100, showed that the increased fluorescence signal originated from the buffer and not from the sedimented vesicles. Apparently, high calcium concentrations caused membrane disruption, allowing leakage of fura-2 from the vesicles. As calcium may cause clustering, rupture and fusion of PS-containing membranes, this finding is not surprising.

The effect of changing the order of successive additions of calcium and annexin V is shown in Fig. 3. In the upper figure, 1 mM calcium was first added to the suspension, and subsequent addition of 150 nM annexin V had no further effects. In the lower figure, annexin V was first added, and subsequent addition of calcium caused a significant increase in fluorescence. The latter experiment was repeated with incubation times of 5 to 20 min for annexin V, and with placental instead of recombinant annexin V, but similar results were obtained. In the lower figure it is also shown that centrifugation had no effect on annexin V-induced fluorescence. Apparently, the

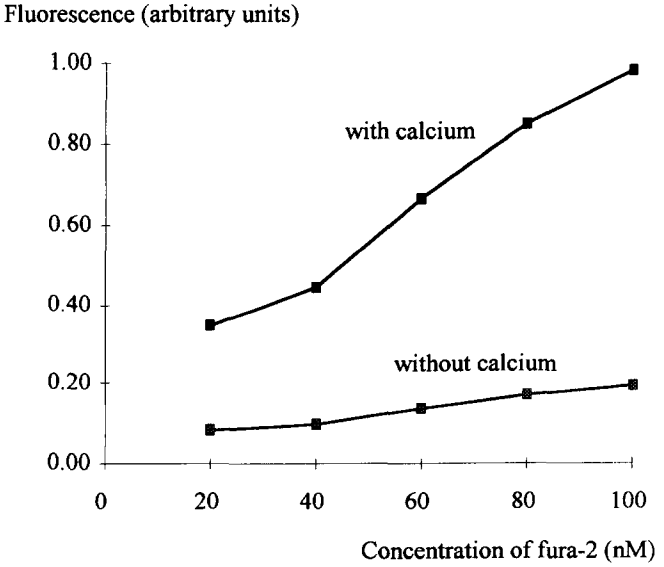



Fig. 1. Effect of calcium (1 mM) and fura-2 concentration on fluorescence at a wavelength of 500 nm. Excitation wavelength was 340 nm.

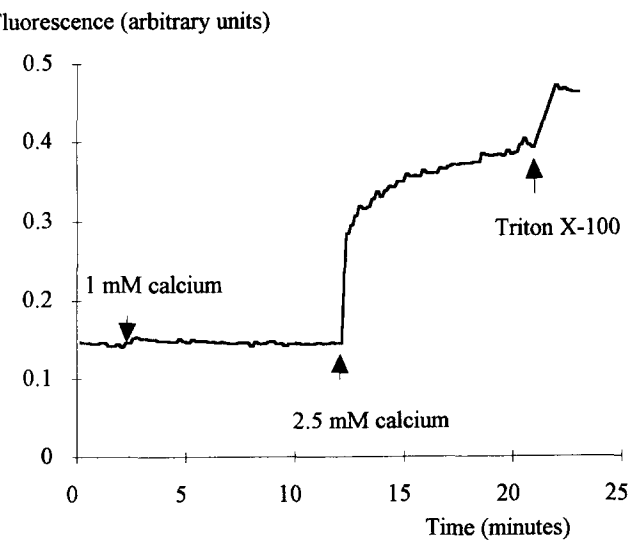



Fig. 2. Effects of calcium on 0.3 mM fura-2-loaded vesicles (LUVs) prepared from a 90% PS/10% PE phospholipid mixture. Calcium and Triton X-100 was added at the indicated times.

sedimented vesicles contributed little to the total signal. The bulk of fura-2 was present in the supernatant and must have leaked out from the vesicles.

The upper part of Fig. 4 shows that bovine serum albumin (5 g/l) has an immediate fluorescent effect, possibly caused by BSA-bound calcium, and also has the labilizing effect of annexin V that only becomes apparent after addition of more calcium. In contrast to the situation for annexin V, however, previous addition of calcium (1 mM) does not protect the membrane from BSA-induced leakage of fura-2 from the vesicles, as follows from the lower part of Fig. 4.

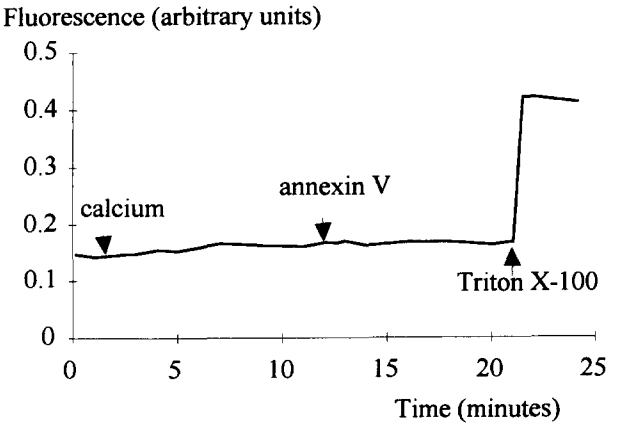
## 4. Discussion

In the presence of calcium, annexin V binds strongly to PS-containing bilayers, forming two-dimensional clusters of 0.22 μg/cm$^2$ of surface mass, as measured by ellipsometry [2,3]. During adsorption, the rate of increase of surface mass equals the transport-limited rate of protein transport from the buffer towards the membrane [3]. This indicates that no significant penetration of the protein into the bilayer occurs because, due to the free lateral mobility of the phospholipids, such penetration would lower the phospholipid mass per unit surface area and the rate of surface mass increase would have been less than the transport limit. Moreover, immediate desorption of annexin V is observed after addition of EDTA, or after lowering the calcium concentration below 50 μM, and the remaining surface mass exactly equals the original mass of the phospholipid bilayer [2,3]. These data demonstrate that the binding of annexin V is fully peripheral, and this has been recently confirmed by ultrastructural analysis of membrane-bound annexin V [8]. Such peripheral binding seems hard to reconcile with channel-forming properties [13].

The membrane interaction of annexin V in the absence of calcium cannot be detected by ellipsometry [2] and this implies that either a minimal amount of protein (<5 ng/cm$^2$) interacts with the bilayer or that penetration occurs, with protein mass

replacing lipid mass. The protein/lipid weight ratio in Fig. 3 is 0.05, whereas this weight ratio is 0.5 for maximal annexin V adsorption on phospholipid bilayers [2], so there is plenty of empty space on the vesicle surface. In spite of this free space, no leak will occur in the presence of calcium. Apparently, the membrane-perturbing interaction will not occur if the calcium-mediated interaction is possible. It was shown that in (calcium-) bound annexin V the N-terminal part of the molecule points away from the lipid surface. Exchange of this part of the molecule by the N-terminus of annexin I (annexin I has stronger membrane-perturbing properties than annexin V [14]) produced a chimeric protein with the core of annexin V that bound equally well to the bilayers as annexin V. In contrast to annexin V, however, the lipid-bound chimeric protein was able to adsorb phospholipid vesicles from the buffer, probably by interaction with the N-terminus [15]. Taken together, these data indicate that the N-terminus of annexin V is involved in the membrane-perturbing interaction.

In contrast to the native protein, the recombinant protein has an unblocked N-terminal alanine causing a slight increase in the pI, from 4.8 to 4.9 [11]. We therefore performed control experiments with annexin V purified from human placenta [1], but the results for the native and recombinant proteins were identical.



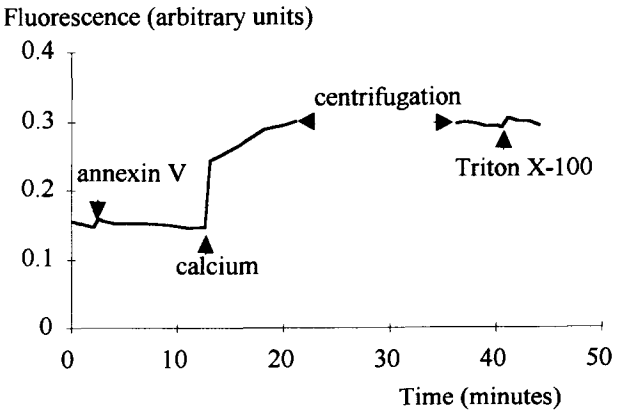
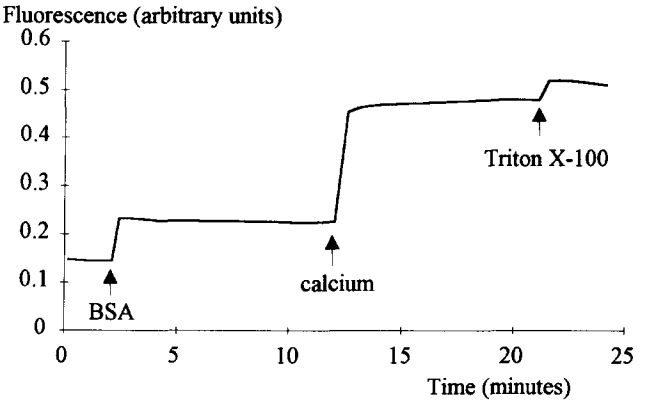




Fig. 3. Effect of the order of successive additions of calcium and annexin V. The upper figure shows that addition of annexin V (150 nM) had no effect after previous addition of calcium (1 mM). The lower figure shows that changing the order of these additions caused a large increase in fluorescence. As also shown in the lower figure, the increased fluorescence could not be eliminated by centrifugation.



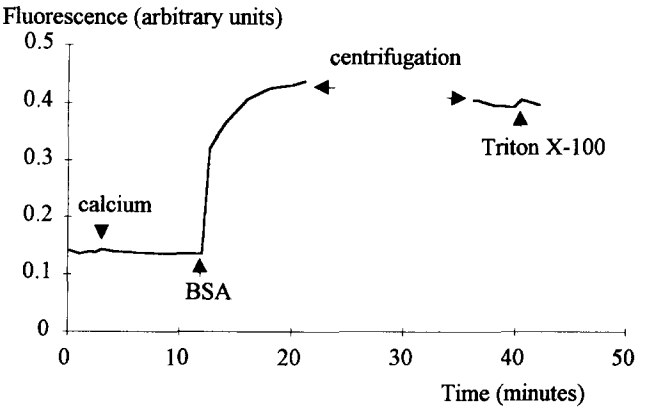




Fig. 4. Effects of addition of bovine serum albumin (BSA) and calcium to fura-2-loaded large phospholipid vesicles (LUVs). Irrespective of the presence of calcium, BSA causes leakage of fura-2 from the LUVs.

The present results confirm recent findings of Hoekstra et al. [14] who demonstrated that, in the absence of calcium, annexin V interacts with PS/PE-vesicles and caused loss of small molecules from the vesicles. Apparently, this interaction is weak and not detectable with ellipsometry [2], but nevertheless it destroys membrane integrity and makes the vesicles leaky. In spite of the use of a very similar assay, our results contrast with those presented by Berendes et al. [6], who found increased fluorescence after addition of annexin V in the presence of calcium. In that study about fifty-fold higher lipid concentrations and a low total calcium concentration of 430 $\mu$M were used. The free calcium concentration was estimated at 250 $\mu$M and this concentration could have been low enough to allow comparison with the results in the absence of calcium from the present study.

In our group, Dr. H. Miedema (unpublished results) has systematically investigated the effect of placental and recombinant annexin V on the electrical conductance of black lipid membranes of the Mueller–Montal type [9,10]. Such membanes have about one million times larger surface areas than membranes on micropipette tips. PS/PE mixtures of increasing PS content, up to 90% PS, and addition of phosphatidylcholine (PC) and cholesterol, were studied. Incubation times with annexin V, in the presence and absence of 1 mM calcium, were varied between 10 and 45 min. However, he was unable to

demonstrate any consistent effect of annexin V on membrane conductivity, although the same bilayers were successfully used to demonstrate the channel properties of the $F_0$ part of beef-heart ATP-synthase [16]. Comparison of these results for planar membranes with the membrane perturbation of LUVs by annexin V in the present study, suggests that the curvature of bilayer surfaces promotes their lability.

Aspecific perturbation of LUVs by proteins was already mentioned in the original publication of Reeves and Dowben [12]. Attempts to stabilize the vesicle suspension with high-molecular mass components succeeded when dextrans or polyethylene glycol was used, but proteins could not be used because they made the vesicles permeable to low molecular mass solutes. Extraction of phospholipids, lyso-phospholipids and fatty acids from the membrane has been described as a mechanism for albumin-mediated membrane damage [17]. Both effects could explain the effects of BSA in the present study.

In conclusion, our results indicate that in the absence of calcium, annexin V exerts an aspecific interaction with LUV membranes that makes the membrane permeable to substances of at least the mass of fura-2 ($M_r$ = 832). In the presence of calcium this interaction does not occur, because of the calcium-mediated and high-affinity interaction of the core of the annexin V molecule with the membrane. Although our experimental conditions were not quite identical to those of Berendes et al. [6], our results raise at least some doubts on the claimed function of annexin V as a voltage-gated calcium channel. It seems as if careful reconsideration is required before a definite function can be ascribed to annexin V.

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